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Use of Randomly Cloned DNA Fragments for Identification of *Bacteroides thetaiotaomicron*

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Randomly cloned fragments of DNA from *Bacteroides thetaiotaomicron* were used as hybridization probes for differentiation of *B. thetaiotaomicron* from closely related *Bacteroides* species. *Hin*dIII digestion fragments of DNA from *B. thetaiotaomicron* (type strain) were inserted into plasmid pBR322 and labeled with [α-^{32}P]dCTP by nick translation. These labeled plasmids were screened for hybridization to *Hin*dIII digests of chromosomal DNA from type strains of the following human colonic *Bacteroides* species: *B. thetaiotaomicron*, *Bacteroides ovatus*, reference strain 3452-A (formerly part of *B. distasonis*), *Bacteroides uniformis*, *Bacteroides fragilis*, *Bacteroides vulgatus*, *Bacteroides distasonis*, *Bacteroides eggerthii*, and reference strain B5-21 (formerly *B. fragilis* subsp. a). Two of the five cloned fragments hybridized only to DNA from *B. thetaiotaomicron*. Each of these two fragments hybridized to the same DNA restriction fragment in five strains of *B. thetaiotaomicron* other than the strain from which the DNA was cloned. One of the cloned fragments (pBT2) was further tested for specificity by determining its ability to hybridize to DNA from 65 additional strains of colonic *Bacteroides*.

There are many important groups of organisms for which no simple method of species identification is available. A good example of such a group is *Bacteroides* species. *Bacteroides* is one of the numerically predominant genera in the bacterial flora of the human colon (16). When colonic *Bacteroides* strains were first studied, they appeared to be so similar phenotypically that most isolates were classified as subspecies of *B. fragilis* (16). Recently, however, the former subspecies of *B. fragilis* have been elevated to species rank on the basis of DNA homology data (6, 9), indicating that these *Bacteroides* species differ significantly from one another at the genetic level. Fermentation tests, which are used routinely for classification of anaerobes, do not readily differentiate some of them, e.g., *B. thetaiotaomicron*, *B. ovatus*, and *B. uniformis* (8). Moreover, the battery of fermentation tests which must be used for identification is cumbersome and time consuming. No simple selective or differential media are available.

Alternative methods for differentiation of some of these species have been proposed. Lambe (12) and Kasper et al. (10) developed fluorescent antiserum which is specific for *B. fragilis*. This antiserum has proven to be useful for identifying strains of *B. fragilis* which have been isolated from clinical specimens. Booth et al. (5) proposed a method based on sensitivity to bacteriophages for identifying *B. fragilis*. This method has the drawback that mutations in the phage receptor could occur or that the capsules which are produced by some *Bacteroides* species could interfere with phage attachment.

Since decisions about what constitutes a species are based on genetic relatedness, the ideal identification procedure would be one which is based directly on DNA-DNA hybridization. Such an identification method would have the advantage of being both highly specific and not dependent on gene expression. Classical methods for determining DNA-DNA homology are too cumbersome to be used for routine identifications because purified chromosomal DNA must be obtained from each of the strains to be tested. Since highly homologous fragments of DNA should provide a simpler and more specific probe than the entire chromosome, we obtained randomly cloned fragments of DNA from the type strain of *B. thetaiotaomicron*, a major colonic *Bacteroides* species, and surveyed these cloned fragments for the ability to hybridize to DNA from strains of *B. thetaiotaomicron* but not to DNA from strains of other colonic *Bacteroides* species. We chose *B. thetaiotaomicron* for this work because it is one of a group of species which are particularly hard to separate on the basis of phenotypic traits. This group includes

TABLE 1. *Bacteroides* strains

Bacteroides species	Strain no.[a]	Homology with *B. thetaiotaomicron* type strain[b] (%)
B. thetaiotaomicron	5482A (ATCC 29148)	(100)
	0633	80
	0940	91
	2808B	93
	3089	75
	3164A	81
B. ovatus	0038 (ATCC 8483)	40
"3452-A"	3452A	36
B. uniformis	0061 (ATCC 8492)	22
B. fragilis, subgroup I	2553 (ATCC 25285)	28
B. fragilis subgroup II	2393	28
B. vulgatus	4245 (ATCC 8482)	12
B. distasonis	4243 (ATCC 8503)	7
"B5-21"	B5-21	21
B. eggerthii	B8-51	23

[a] Unless otherwise noted, strain numbers are VPI numbers.
[b] Data from reference 9.

B. ovatus and *B. uniformis*, both of which have a DNA cross-homology with *B. thetaiotaomicron* of 30 to 40% (9). *B. eggerthii* and two unnamed DNA homology groups, "3452-A" (formerly part of *B. fragilis* subsp. *distasonis*) and "B5-21" (formerly *B. fragilis* subsp. a) are also very closely related to *B. thetaiomicron* (9). In this report, we describe the isolation of two randomly cloned fragments of chromosomal DNA which are specific for *B. thetaiotaomicron*.

MATERIALS AND METHODS

Bacterial strains. *B. thetaiotaomicron* (VPI 5482, ATCC 29148) was the source of the DNA fragments used to construct the recombinant plasmids which were tested as hybridization probes. The strains of *Bacteroides* used in the initial screening of these cloned fragments are listed in Table 1. Also given in Table 1 are the percent DNA-DNA homologies, obtained previously by Johnson (9), berween each of these strains and *B. thetaiotaomicron* strain VPI 5482A. These and other *Bacteroides* strains used in these experiments were obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University (VPI), Blacksburg, and were included in the original DNA homology studies of Johnson (9). The origin of each strain is described in that study. All strains are unrelated. *Escherichia coli* K-12 strain C600-SF8 (20) was used as the recipient for transformation with the recombinant plasmids.

Preparation of DNA. Cultures were grown to an optical density (650 nm) of 0.6 to 0.8 in peptone-yeast extract medium (8) which contained glucose (0.5%) as the source of carbohydrate. Chromosomal DNA was extracted and purified by the method of Saito and Miura (18). Fragments of *Bacteroides* DNA were generated by digestion of 10 μg of chromosomal DNA with 20 U of *Hin*dIII at 37°C for 16 h. pBR322 (4) was similarly digested with *Hin*dIII and then was ligated with the *Bacteroides* fragments, using T4 DNA ligase (13). *Hin*dIII was obtained from Bethesda Research Laboratories. T4 DNA ligase was purified by an unpublished procedure (R. Gumport, personal communication).

E. coli C600-SF8 was transformed with the ligation mixture (15), and colonies which were resistant to ampicillin and susceptible to tetracycline were isolated and screened for plasmids by the procedure of Birnboim and Doly (2). Five colonies which contained plasmids larger than pBR322 were chosen for further studies. Recombinant plasmids were isolated (15), and the molecular weights of the inserted fragments were determined by agarose gel (19, 21) or polyacrylamide gel (3) electrophoresis after digestion of the recombinant plasmid with *Hin*dIII.

Screening of cloned fragments. Recombinant plasmids were labeled with [α-^{32}P]dCTP by nick translation (7). pBR322, similarly labeled, was used to confirm that there was no hybridization between the cloning vector and the *Bacteroides* species included in the survey. [α-^{32}P]dCTP (>600 Ci/mmol) was purchased from New England Nuclear Corp. The specific activity of the labeled plasmids was 15 to 25 μCi per μg of DNA.

Purified chromosomal DNA from the *Bacteroides* strains listed in Table 1 was digested by incubating 20 to 30 μg of DNA with 20 U of *Hin*dIII at 37°C for 8 to 12 h. A second addition of 10 U of *Hin*dIII was made, and the mixture was incubated for another 8 h at 37°C. For the hybridization experiments, approximately 2 μg of *Hin*dIII-digested chromosomal DNA was subjected to electrophoresis on a 1% (wt/vol) agarose gel. After electrophoresis, the DNA was transferred to nitrocellulose paper (Millipore HAWP) by the Southern blot procedure (7). The DNA which had been transferred to the nitrocellulose paper was hybridized with the labeled plasmid in 50% formamide (procedure 4b in reference 7). After hybridization for 48 h at 42°C, the blot was washed twice for 20 min at 55°C in 250 ml of 2× SSPE–0.2% sodium dodecyl sulfate (SDS) and twice in 0.2× SSPE–0.2% SDS. (SSPE is 0.18 M NaCl–10 mM sodium phosphate buffer [pH 7.0]–1 mM EDTA). Hybridization of the labeled plasmid to DNA trapped on the nitrocellulose paper was detected by autoradiography.

One of the recombinant plasmids which appeared to be specific for *B. thetaiotaomicron* was further tested for hybridization to 65 additional colonic *Bacteroides* strains. A modified form of the colony hybridization procedure was used for this screening. Each strain was grown in peptone-yeast extract-glucose medium (8) to an optical density (650 nm) of 0.9 to 1.0. The bacteria in 0.5 ml of culture (ca. 2×10^9 CFU) were collected onto a 22-mm-diameter portion of a strip of nitrocellulose paper by vacuum filtration. Each strip of nitrocellulose paper contained duplicates of *B. thetaiotaomicron* VPI 5482 (positive control), uninoculated medium (negative control), and the strains being tested. After hybridization with labeled plasmid, each of the segments of filter was cut out, placed in scintillation vials, digested (14), and counted by liquid scintillation spectroscopy in Aquasol II.

RESULTS

Screening of cloned fragments for specificity. When recombinant plasmids which contained *B. thetaiotaomicron* DNA were hybridized with blots of *Bacteroides* DNA, they hybridized in all cases with the predicted *Hin*dIII fragment from *B. thetaiotaomicron* (Fig. 1a to d). Two of the recombinant plasmids (pBT2 and pBT5) contained fragments which hybridized with *B. thetaiotaomicron* but not with DNA from any of the other *Bacteroides* species (Fig. 1a and b, lanes 2 to 11). No cross-hybridization was seen, even when a lower stringency wash procedure was used. These plasmids also hybridized with DNA from five strains of *B. thetaiotaomicron* other than the type strain. The restriction fragments from all of these strains were the same size (Fig. 1a and b, lanes 12 to 17).

One of the recombinant plasmids (pBT3) hybridized with all of the *Bacteroides* species tested except *B. distasonis* (Fig. 1c, lanes 2 to 11). The fact that all of the cross-hybridizing fragments from other species had different sizes from that of the fragment from *B. thetaiotaomicron* indicates that they differed by at least one *Hin*dIII site. Even among different strains of *B. thetaiotaomicron*, there were differences in the sizes of the fragments which hybridized to pBT3 (Fig. 1c, lanes 12 to 17).

Two of the recombinant plasmids (pBT1 and pBT4) hybridized only to species which were very closely related to *B. thetaiotaomicron* (Table 1). For example, pBT1 hybridized to DNA from both *B. ovatus* and reference strain 3452-A, as well as to DNA from *B. thetaiotaomicron* (Fig. 1d, lanes 2 to 4). The cross-hybridizing restriction fragment from reference strain 3452-A was larger than the restriction fragment from *B. ovatus* or from *B. thetaiotaomicron*. pBT1 hybridized to all six strains of *B. thetaiotaomicron*, and all of the cross-hybridizing fragments were the same size (Fig. 1d, lanes 12 to 17). The other recombinant plasmid (pBT4, not shown) cross-hybridized with a restriction fragment from reference strain 3452-A as well as with the predicted fragment from *B. thetaiotaomicron*. The cross-hybridizing fragment from reference strain 3452-A was larger than the fragment from *B. thetaiotaomicron*. The sizes of the different cloned fragments from *B. thetaiotaomicron* and their specificities are summarized in Table 2. None of these five fragments cross-hybridized with any of the other fragments.

Sensitivity of the method. To estimate the potential sensitivity of this method for detecting *B. thetaiotaomicron*, we loaded different amounts of *Hin*dIII-digested *B. thetaiotaomicron* DNA into various wells of an agarose gel. Enough *Hin*dIII-digested DNA from reference stain 3452-A was added to each well so that the total DNA in the well was constant (1.6 μg). After electrophoresis and transfer to nitrocellulose paper, the DNA was hybridized with labeled pBT2. The fragments could be detected in as little as 50 ng of *B. thetaiotaomicron* DNA (Fig. 2). Since 10^9 cells of *B. thetaiotaomicron* contain 4 to 5 μg of DNA (11), this means that at least 10^7 cells of *B. thetaiotaomicron* would be required to produce a hybridization reaction which is detectable by autoradiography. Similar results were obtained with pBT5 (data not shown).

Further screening by modified colony hybridization technique. From the results of the experiments described above, two recombinant plasmids, pBT2 and pBT5, appeared to be specific for *B. thetaiotaomicron*. As a further test of the specificity of pBT2, we screened 65 additional strains of colonic *Bacteroides* for hybridization with pBT2. For this screening, we used a modification of the colony hybridization method in which approximately 1×10^9 to 2×10^9 cells were concentrated onto 22-mm-diameter areas of a nitrocellulose strip by vacuum filtration. Hybridization was measured by liquid scintillation counting (Table 3). The amount of radioactivity which was bound to the positive control (*B. thetaiotaomicron* VPI 5482) varied somewhat from one hybridization experiment to another, due to differences in the age of the probe and the number of cells on the filter. However, the amount of radioactivity which was bound to strains of *Bacteroides* species other than *B. thetaiotaomicron* was consistently 10% or less than the amount bound to *B. thetaiotaomicron* 5482B. Strains of *B. thetaiotaomicron* other than 5482A bound 65 to 146% as much radioactivity as 5482A.

Detecting *B. thetaiotaomicron* in a mixed culture. To determine whether *B. thetaiotaomicron* could be detected in a mixed culture which contained a closely related *Bacteroides* species, a culture of *B. thetaiotaomicron* was mixed with a culture of reference strain 3452-A (VPI 3452A) in different ratios so that the total number of bacteria in the 0.5 ml placed on the nitrocellulose filter was 1.2×10^9. When 1.2×10^9, 0.6×10^9, 0.3×10^9, and 0.1×10^9 cells of *B.*

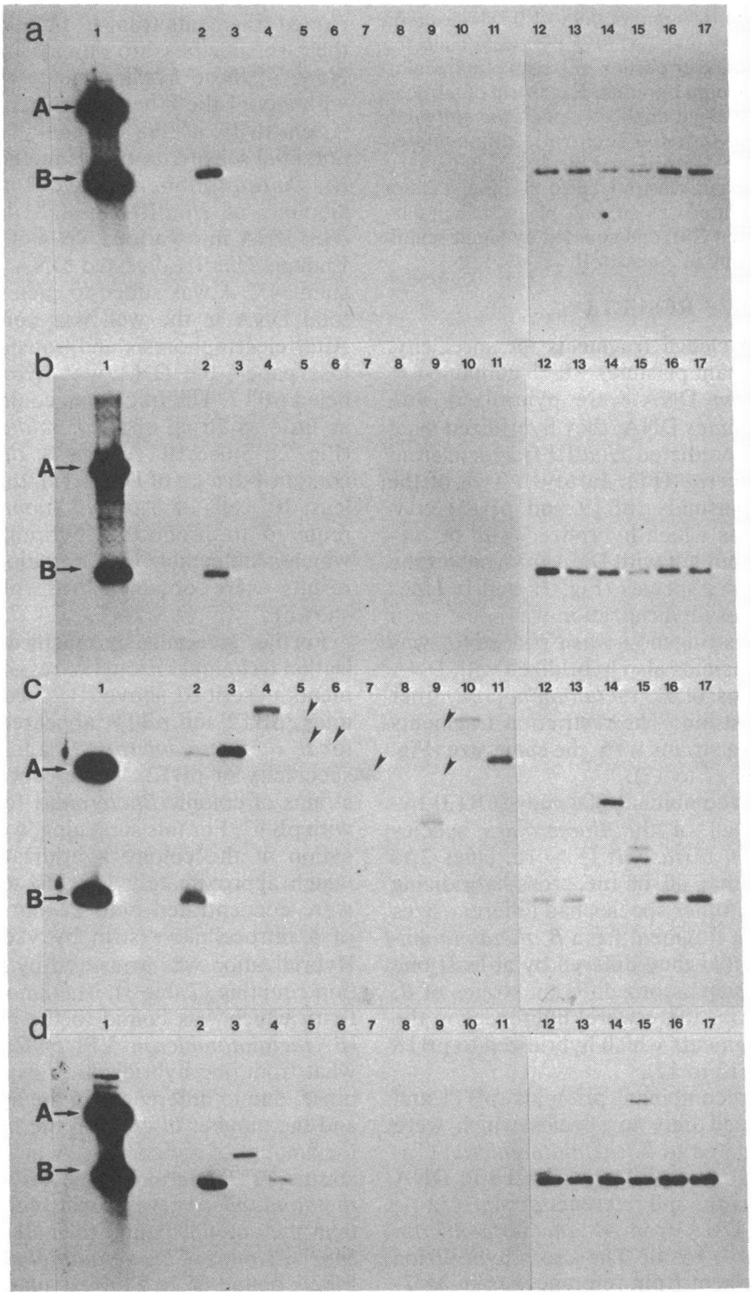

FIG. 1. Autoradiograms of Southern blots of DNA from various *Bacteroides* strains with ^{32}P-labeled pBT2 (panel a), pBT5 (panel b), pBT1 (panel c), and pBT3 (panel d). Lane 1 contained a *Hin*dIII digest of the plasmid which was used as the hybridization probe. Arrows indicate the migration distance of digested pBR322 (A) and the insert (B). Lanes 2 to 11 contained, respectively, *Hin*dIII digests of DNA from the following strains: *B. thetaiotaomicron* (VPI 5482), reference strain 3452-A (VPI 3452A), *B. ovatus* (VPI 0038), *B. uniformis* (VPI 0061), *B. fragilis* subgroup I (VPI 2553), *B. fragilis* subgroup II (VPI 2393), *B. distasonis* (VPI 4243), *B. vulgatus* (VPI 4245), *B. eggerthii* (VPI B8-51), and reference strain B5-21 (VPI B5-21). Lanes 12 to 17 contained, respectively, *Hin*dIII digests of DNA from *B. thetaiotaomicron* strains VPI 5482, VPI 0633, VPI 0940, VPI 2808B, VPI 3164, and VPI 3089. Lane 1 contained 20 to 50 ng of DNA, and lanes 2 to 17 each contained 1,500 to 2,000 ng of DNA. Exposure time was 18 to 24 h. Arrows indicate the positions of bands which were detected when the autoradiogram was exposed for 1 week.

TABLE 2. Size of insert and specificity of the recombinant plasmids which were surveyed

Plasmid	Approx size of insert (kbp)[a]	Positive hybridization with[b]:
pBT1	2.55	*B. thetaiotaomicron*, *B. ovatus*, and reference strain 3452-A
pBT2	2.28	*B. thetaiotaomicron* only
pBT3	0.79	*B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, *B. fragilis*, *B. vulgatus*, *B. eggerthii*, reference strains 3452-A and B5-21
pBT4	0.41	*B. thetaiotaomicron* and reference strain 3452-A
pBT5	1.32	*B. thetaiotaomicron* only

[a] Size in kilobase pairs (kbp) as determined from migration distance on agarose or acrylamide gels (see the text).

[b] From Fig. 1.

thetaiotaomicron were in the mixture being tested, the radioactivity on the filter (background subtracted) was 7,500, 4,000, 1,800, and 500 cpm, respectively. Measurements were done in triplicate, and variation was less than 20% of the mean. Thus, *B. thetaiotaomicron* could be detected in a mixed culture, even when it represented only 10% of the total number of bacteria on the filter. The limit of detection by liquid scintillation counting appeared to be approximately 10^8 bacteria.

DISCUSSION

Hybridization with labeled cloned DNA has been widely used to compare antibiotic resistance determinants and amino acid biosynthetic genes from different organisms. Recently, Moseley et al. (17) have also used a ^{32}P-labeled plasmid which carried genes for *E. coli* enterotoxins to detect enterotoxigenic strains of this organism. Since we have used randomly cloned fragments of chromosomal DNA rather than cloned genes which code for a particular phenotype, our approach is not restricted to organisms on which extensive genetic and metabolic research has been done. Our approach can even be used to identify organisms whose DNA is not expressed in *E. coli*.

Two of the five cloned fragments which were screened in this study hybridized to DNA from *B. thetaiotaomicron* but not to DNA from closely related *Bacteroides* species. These fragments (pBT2 and pBT5) hybridized to restriction fragments of the same sizes in six different strains of *B. thetaiotaomicron*. Thus, the fragments appear to be species specific rather than strain specific. For this study, we labeled our fragments with ^{32}P. This isotope gives high sensitivity but has a short half-life. As fluorescent nucleotides or methods of labeling DNA with fluorescent compounds become available, it may be possible to dispense with ^{32}P in favor of a more stable form of labeled fragment.

Labeled pBT2 hybridized to DNA from bacteria collected on nitrocellulose paper and lysed with NaOH. Thus, when homologous fragments are used as probes, it is not necessary to extract and purify DNA from the strain to be tested, as must be done if the entire chromosome is used in the hybridization. The cloned fragments described in this report could also detect *B. the-*

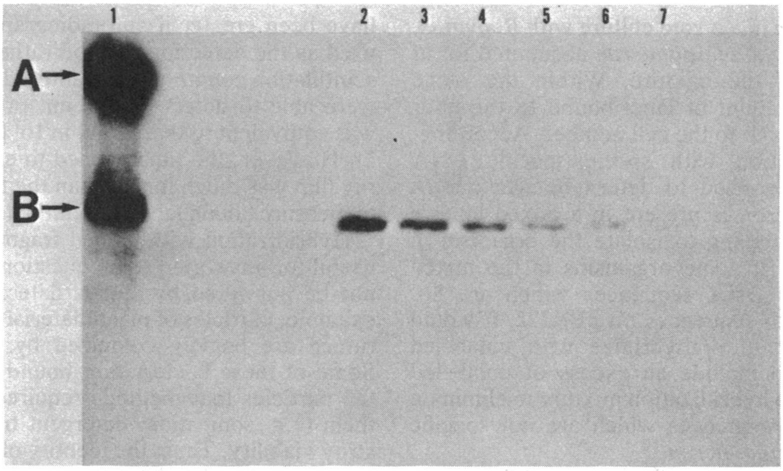

FIG. 2. Hybridization of ^{32}P-labeled pBT2 DNA to different concentrations of *Hin*dIII-digested DNA from *B. thetaiotaomicron* (VPI 5482). Lane 1 contains 20 ng of *Hin*dIII-digested pBT2 as a positive control. Lanes 2 to 7 contain the following amounts of *Hin*dIII-digested DNA from *B. thetaiotaomicron*: 2, 1,600 ng; 3, 800 ng; 4, 400 ng; 5, 200 ng; 6, 100 ng; 7, 50 ng. Enough *Hin*dIII-digested DNA from reference strain 3452-A (VPI 3452A) was added to each well to make a total of 1,600 ng of DNA in each well. Exposure time was 36 h.

TABLE 3. Survey of 74 strains from 8 colonic *Bacteriodes* species for hybridization with pBT2

Species and strain	^{32}P-labeled pBT2 DNA bound (% that bound to 5482A)[a]	Species and strain	^{32}P-labeled pBT2 DNA bound (% that bound to 5482A)[a]	Species and strain	^{32}P-labeled pBT2 DNA bound (% that bound to 5482A)[a]
B. thetaiotaomicron		*B. fragilis*		"3452-A"[c]	
5482A	100	Group I[b]		3452A	1
0633	84	2553	3.5	8608	0.5
0940	65	0479	0.5	B6-11	2
2808B	71	1522	1.5	C7-8	0.5
3089	121	1582	1.5	C10-2	0.5
3164	139	2556	0.5	C14-3	0
3443	146	3277	0	*B. distasonis*	
5951	120	4361	0.5	4243	0.5
7330	104	4509B	0.5	0052	1
J19-34	116	4517	2	6050	1
C11-15	143	Group II		B1-20	1
B. ovatus		2392	1	C14-2	1
Group I[b]		2552	2.5	C18-7	1.5
0038	1	2647-J2	2.5	C19-17	1
0435	3	3392	1.5	C30-45	1
2828	1.5	4076	0.5	T3-25	1
3049	2.5	4117	1	S6A-50	0.5
4101	6	4225	0.5	*B. vulgatus*	
C1-45	1	A11-24B	0.5	4245	1
C16-22	2.5	*B. uniformis*		2277	0.5
R3-39	0	0061	2	2365	1.5
Group II[b]		0909	0.5	3776	0
C2-26	4.5	3537	0.5	4025	1
B4-11	4	C7-17	1.5	4506	0.5
"B5-21"[c]		C20-25	1.5	5710	1
B5-21	0	R5-33	3	6186	1.5
C8-19	0	T1-1	1	6598B	1.5
C51-6	0.5			C1-13	1

[a] [Cpm bound to strain being tested (minus background)/cpm bound to *B. thetaiotaomicron* 5482A (minus background)] × 100. Background = cpm bound to uninoculated media. Amount of radioactivity bound to 5482A ranged from 7,500 to 12,300 cpm; background was 100 to 250 cpm. Each value is the mean of four determinations.
[b] DNA-DNA homology subgroup (9).
[c] Unnamed DNA homology group (9).

taiotaomicron in a mixed culture with *B. ovatus*, as long as *B. thetaiotaomicron* accounted for at least 10% of the mixture. Within the range tested, the amount of label bound to the filter was proportional to the cell number. Accordingly, hybridization with species-specific DNA probes can be used to determine whether *B. thetaiotaomicron* is present in a mixed culture without first having to isolate the organism in pure culture. If some organisms in the mixed culture carry DNA sequences which are homologous with sequences on pBR322, it would be necessary to prehybridize with unlabeled pBR322 or to include an excess of unlabeled pBR322 in the hybridization mixture to eliminate signals from sequences which are not specific for *B. thetaiotaomicron*.

The main limitation of this method for detection and identification of *B. thetaiotaomicron* is its sensitivity. In our modified colony hybridization procedure, the limit of detection was approximately 10^8 bacteria. The sensitivity would have been greater if autoradiography had been used as the detection method rather than liquid scintillation counting. With autoradiography, we were able to detect an amount of DNA which was equivalent to that found in 10^7 bacteria (Fig. 2). However, the time needed to expose the X-ray film was much longer than the time required to measure bound label by scintillation counting.

Hybridization with cloned fragments may be useful for answering some questions which cannot be answered by standard techniques. For example, particles of plant material in the bovine rumen are heavily colonized by bacteria (1). Some of these bacteria are bound so tightly to the particles that methods required to dislodge them (e.g. sonication, detergent treatment) destroy viability. Thus, the identity of these organisms can only be determined tentatively from their morphology in electron micrographs and from the results of in vitro colonization experiments. The identity of these organisms could be established directly by obtaining specific cloned

DNA fragments from organisms thought to colonize particles and hybridizing these fragments with DNA eluted from colonized particles by detergents or NaOH.

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